Title: METHODS FOR PRODUCING AND TRANSFORMING CASSAVA PROTOPLASTS

This is a Continuation-in-Part of application USSN 09/180,481, the contents of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Genetic modification or transformation is the technique wherein one or a few gene(s) are added to a commercial interesting genotype or clone. In principle a successful transformation system requires an efficient system where new plants are formed from specific plant parts (stem, leaf, node and so on) or from specific tissue (somatic embryos, embryogenic callus, friable embryogenic callus) induced on specific plant parts or from protoplasts (single cells without cell wall) derived of these parts or from the specific tissue, a system to transfer DNA molecules to the plant's parts or protoplasts and a system to select tissue and plants which contain and express the introduced gene(s). In principle protoplasts are the most ideal system for DNA delivery. They can be cultured as single cells that produce multicellular colonies from which plants develop. Plants derived from protoplasts are generally clonal in origin. This provides a useful tool for any transformation system, because it will eliminate chimerism in transgenic plants.

Cassava is very recalcitrant for plant regeneration of protoplasts. There is only one report of shoot regeneration from protoplasts of cassava (Shahin and Shephard, 1980). They used well expanded leaves for the isolation of protoplasts. Despite considerable efforts, plant regeneration from protoplasts (isolated from leaves, sterns, and roots) has never been repeated since then (Anonymus, 1985; Nzoghe, 1991; Anthony *et al.*, 1995, Sofiari, 1996). A logical approach was to use tissues which contain embryogenic cells. Such cells are found in the apical meristems, young leaves or somatic embryos cultured on auxin supplemented media (Stamp and Henshaw, 1987a; Raemakers *et al.*, 1993a). However, protoplasts isolated from these tissues

gave in the best case green callus and adventitious roots (Sofiari, 1996). Recently, a new type of somatic embryogenesis was developed. In this *in vitro* system the embryos do not develop beyond the (pre-)globular stage and the embryogenic callus is highly friable (Taylor *et al.*, 1995). Transfer of this friable embryogenic callus (FEC) to liquid medium resulted in a suspension-like culture. In leek (Buitenveld and Creemers, 1994), petunia (Power *et al.*, 1979), rice (Kyozuka *et al.*, 1988), sugarcane (Chen, *et al.*, 1988), and wheat (Chang *et al.*, 1991) such cultures were an excellent source for protoplast regeneration. We have now found that in cassava FEC is the only tissue from which protoplasts van be isolated which are able to regenerate into plants sofar.

SUMMARY OF THE INVENTION

Thus the present invention provides a method for producing protoplasts of cassava or a closely related species, which protoplasts are capable of regeneration into plants, comprising producing friable embryogenic callus from explants of cassava or a closely related species and isolating protoplasts from said friable embryogenic callus. It appears, as will be described below, that for obtaining suitable protoplasts the culture in solution of the FEC is quite important. Therefore the present invention further provides a method wherein the friable embryogenic callus is subjected to culture in a liquid medium.

Protoplasts are preferably produced by subjecting plant cells to enzymatic breakdown of the cell walls. The invention thus provides a method wherein a mixture of cell wall degrading enzymes, such as a cellulase, a pectolyase and/or a macerozyme are used to produce protoplasts.

It also appears that the method according to the invention works best when the plants from which explants are to be taken are pretreated. Therefore the invention provides a method wherein the plants from which explants are taken are pretreated with an auxin as described below.

On the explants preferably embryogenesis is induced resulting in an invented method wherein the friable embryogenic callus is produced from primary, secondary or cyclic embryogenic tissue. The reason is explained in the detailed description. Protoplasts obtainable by a method as disclosed above are also part of the invention.

An important reason for wanting to have protoplasts which can be regenerated into plants is of course that protoplasts can be easily transformed or transduced or provided with additional genetic information by any other suitable method. Thus one is now able to provide cassava plants or closely related species with genetic material of interest. The invention thus also provides a method for transforming (defined as providing with in any suitable manner) a protoplast of a cassava or a closely related species by providing said protoplast with additional genetic information through infection by a bacterium comprising said additional genetic information such as Agrobacterium tumefaciens, by electroporation or chemical poration providing a vector comprising said additional genetic information or by particle bombardment wherein the particles are coated with the additional genetic information, wherein a protoplast obtainable from friable embryogenic callus is transformed. The invention also encompasses transformed protoplasts obtainable by such a method.

Below a short introduction is given on the usefulness of transforming plants, such as cassava.

Application of plant gene technology encompasses a multitude of different techniques ranging from isolation of useful genes, their characterization and manipulation, to the reintroduction of modified constructs into the plants (Lonsdale, 1987). Plant gene technology will catalyze progress in plant breeding, as is exemplified by a few examples of transgenic crops like rice (Chen *et al.*, 1987; Shimamoto *et al.*, 1989), maize (Gordon-Kamm *et al.*, 1990; Vain *et al.*, 1993), wheat (Marks *et al.*, 1989), and potato (De Block, 1988; Visser *et al.*, 1989). Rapid progress in gene technology has

allowed insight into the complex molecular mechanism of plant pathogen recognition and the natural defense strategies of host plants. This technology can also be used for controlled and efficient identification of desirable genotypes, far beyond the possibilities of classical breeding.

For instance electroporation of protoplasts derived from suspension cultures led to the transformation of maize (Rhodes *et al.*, 1988), rice (Toriyama *et al.*, 1988) and orchardgrass (Horn *et al.*, 1988).

Successful attempts have been made to improve resistance against pathogenic viruses like tobamovirus in tobacco (Powel Abel *et al.*, 1986), potexvirus in potato (Hoekema *et al.*, 1989) and in papaya (Fitch *et al.*, 1992). In the above examples the introduced trait was based on expressing of single genes that are coding for the coat protein. In cassava, African cassava mosaic virus (ACMV) and cassava common mosaic virus (CCMV) may be controlled by the coat protein-mediated resistance technique (Fauquet *et al.*, 1992). The genes encoding key enzymes of cyanogenesis have been cloned (Hughes *et al.*, 1994) which makes manipulation of cassava cyanogenesis by genetic transformation using the antisense approach feasible. Another embodiment of the invention is the manipulation of starch in the cassava tubers.

Thus the present invention provides a transformed protoplast wherein the additional genetic information comprises an antisense construct, particularly one wherein the antisense construct is capable of inhibiting the amylose synthesis pathway.

A protoplast cannot grow in the field, nor can it be harvested. Though protoplasts are necessary for transformation, it must be possible to regenerate said protoplasts into embryos and/or plants. This is a very important embodiment of the invention, because cassava has been shown to be difficult to regenerate from protoplasts. The detailed description explains how this may be achieved. For further information reference is made to the thesis written by E. Sofiari titled Regeneration and Transformation of Cassava (Manihot Esculenta Crantz.), a copy of which is enclosed with the present application, which is

incorporated herein by reference. Thus the invention provides a method for regenerating plants from protoplasts wherein a protoplast according to the invention is induced to produce an embryo, which embryo is consequently induced to produce a plant.

The plants obtainable by said method are also part of the invention, in particular plants wherein the tubers contain essentially no amylose.

In addition, the invention provides a method for obtaining starch from the tubers of said cassava plants. The method can be carried out in a manner essentially similar to a method for isolating starch from potato tubers. In one embodiment of the invention, the method comprises the steps of washing the tubers, followed by grating and milling them. Subsequently, the starch is separated from fibers and juice in separators, e.g. centrifuges or hydrocyclones. The isolated starch may then be sieved, washed and dried. Washing may be carried out in hydrocyclones. Drying may be carried out in vacuum filters and drying towers.

The starch obtained from cassava tubers having an increased amylopectin content is also part of the invention. This starch preferably contains essentially no amylose, by it is meant that it preferably has an amylopectin content of at least 95 wt.%, more preferably at least 98 wt.%, based on the (dry substance) weight of the starch. In addition, the starch has a number of distinct properties, such as molecular weight, intrinsic viscosity, particle size, and chain length distribution, which will be further elucidated below.

DETAILED DESCRIPTION OF THE INVENTION

Initiation of FEC

The procedure to obtain FEC is outlined in Figure 1. It starts with the induction of primary embryos. Primary embryos are formed in a two step procedure. In the first step explants are cultured on medium supplemented

with salts and vitamins (preferably Murashige and Skoog (1962)), a carbohydrate source (for example 20 g/l sucrose) and an auxin (*e.g.* 1-8 mg/l picloram, or dicamba or 2,4-D) for the initiation of embryos. After 10 to 15 days on this first medium bipolar torpedo shaped embryos are formed. Torpedo shaped embryos possess a clear hypocotyl and cotyledon primordia. After transfer of the explants with torpedo shaped embryos to a step 2 medium (the same medium as step 1 but without an auxin, with eventually the addition of cytokinins) the torpedo shaped embryos become mature. Mature embryos possess large green cotyledons.

Zygotic embryos (Stamp and Henshaw 1982; Konan *et al.*, 1994), young leaf explants or apical meristems (Stamp and Henshaw, 1987a; Szabados *et al.*, 1987; Mroginsky and Scocchi, 1993; Raemakers 1993a; Narayanaswamy *et al.*, 1995) and floral tissue (Mukherjee, 1995, Woodward and Puonti-Kaerlas, 2001) can be used to obtain primary embryos. In this way many different genotypes were evaluated for their ability to form primary embryos. In this protocol primary somatic embryos were only formed after culture on solid medium and never after culture in liquid medium. Furthermore, somatic embryos (primary) were only observed if the auxins Picloram, Dicamba or 2,4-D were used and not with IAA, IBA or NAA.

In the presently used protocol there is genotypic variation in the number of mature embryos formed per cultured explant. The genotypes M.Col1505, M.Col22 and Gading gave the highest numbers of mature embryos per cultured leaf explant (ME/CLE). However, the number of mature embryos formed was low. In M.Col22 a maximum of 22% of the leaf explants isolated from *in vitro* grown plants and cultured on a step 1 medium with 4 mg/l 2,4-D, formed ME with a maximum number of 0.8 ME/CLE. On a step 1 medium with 8 mg/l 2,4-D a maximum of 49% of the leaf explants formed ME with a maximum number of 3.5 ME/CLE. Higher 2,4-D concentrations did not further improve the embryogenic capacity of explants.

In an attempt to improve the capacity of leaf explants to produce primary somatic embryos, donor plants were grown under different conditions. Growth of *in vitro* donor plants under different light regimes (8, 12, 16 or 24 hours) had no influence on the embryogenic response. However, a reduction of the light intensity had a positive effect. The best results were obtained with leaf explants isolated of donor plants grown at 8 $\mu Em^{-2}s^{-1}$ and cultured on a step 1 medium.

Other investigators have shown that in certain genotypes, Dicamba (1-66 mg/l) and Picloram (1-12 mg/l) are superior to 2,4D for inducing primary embryogenesis (Ng 1992; Sudarmonowati and Henshaw, 1993; Taylor and Henshaw, 1993). Mathews *et al.* (1993) improved the efficiency of primary embryogenesis in the genotype M.Co11505 by transferring explants after 15 days of step 1 medium to a growth regulator-free medium supplemented with 0.5% charcoal. On this medium maturation was improved and as a result the number of mature embryos increased from 0.4 in the control to 3,4 ME/CLE. The best results were obtained if donor plants were pretreated with auxins as 2,4-D or picloram or Dicamba. For this, plants were grown in liquid MS20 medium and supplied with the auxin (final concentration 8 mg/l) after 12 days of growth. Two days later leaf explants were isolated of the donor plants and cultured on step 1 medium with 8 mg/l 2,4-D, picloram or Dicamba. In the clone M.Col22 this resulted in a production of 9.4 ME/CLE. This was significantly higher than in the H_20-treated control-plants, where 3.5 ME/CLE were produced (Table 3).

The general applicability of the auxin pretreatment was tested on several different genotypes. Without a pretreatment of donor plants two genotypes formed ME and at low frequency. After a pretreatment of donor plants, leaf explants of almost all genotypes formed ME.

Eventually we were able to obtain mature primary somatic embryos from 24 of the 28 tested genotypes (Table 1, except TMS30221, TMS30001, TMS30572 and Sao Paolo). These data suggest that almost all genotypes in

cassava can undergo somatic embryogenis and up till now it has been shown that in more than 60 genotypes it has been shown that they can undergo primary somatic embryogenesis (Thro et al., 1999).

Primary somatic embryos derived from zygotic embryos and from leaves have been used as explants to initiate secondary embryos (Stamp and Henshaw, 1987b; Szabados *et al.*, 1987; Mathews *et al.*, 1993; Raemakers et *al.*, 1993bc; Luong *et al.*, 1995). Continuous culture of somatic embryos on auxin supplemented medium resulted in a cyclic system of somatic embryogenesis. The way of subculturing somatic embryos for secondary embryogenesis seemed to influence the morphology of the embryogenic tissue. Clumps of somatic embryos recultured monthly on solid 2,4D containing medium in the dark developed into finger-like embryo initials formed on the top of older embryos. The embryos did not pass the torpedo-shaped stage.

Further development occurred if the clumps with embryos were transferred to step 2 medium in the light (Szabados 1987). Normally mature somatic embryos were cultured in step 1 medium in the light and twenty days later the explants were transferred to step 2 medium for maturation. In this system embryos developed to maturity and mature embryos with large green cotyledons were used to start a new cycle of embryogenesis whereas in the system of others torpedo shaped embryos were used to start a new cycle of secondary somatic embryogenesis.

This system of multiplication of mature embryos has been tested in 14 **20** of the in Table 1 mentioned genotypes. Despite the fact that in most genotypes only a few mature primary embryos ware available, all genotypes, except one, gave new mature embryos after culture on 2,4-D supplemented medium, in a much higher frequency as observed for primary somatic embryogenesis (Raemakers et al.1993b,c, 2000, 2001, Sofiari et al., 1996). Embryogenicity was maintained by regular subculture of mature embryos for more than one year (Szabados *et al.*, 1987; Mathews *et al.*, 1993; Raemakers, 1993). New somatic embryos ware formed both in liquid and solid medium. In

all the genotypes it was observed that in liquid medium more embryos ware formed than in solid medium and that fragmentation of embryos before the start of a new cycle of secondary somatic embryogenesis increased the production compared to whole embryos. In for example M.Col22 whole embryos cultured on solid medium produced 8 embryos per cultured embryo, whereas fragmented embryos cultured in liquid medium produced 32 embryos per cultured embryo (Raemakers *et al.*, 1993c). Not only, 2,4-D, Picloram and Dicamba, but also NAA had the capacity to induce secondary embryogenesis. IBA and IAA did not induce secondary embryogenesis. NAA has been used successfully in Adira 1, Adira 4, Gading, line 11, M.Col22, M.Col1505, TMS90853 and Gading (Sofiari, 1996). In general more mature embryos were produced in NAA supplemented medium than in 2,4-D, Picloram or Dicamba supplemented medium. Furthermore, the development of NAA induced embryos was faster than with 2,4-D, Dicamba or Picloram. Shortening the culture duration has a beneficial effect, particularly, when operating on a large scale.

Histologically, the by 2,4-D newly induced secondary embryos were attached vertically to the explants whereas those by NAA were horizontally.

Some scientists have still a problem in obtaining embryogenic cultures in certain genotypes of cassava (Mroginski and Scocchi, 1992; Taylor *et al.*, 1992; Narayanaswamy *et al.*, 1995; Sudarmonowati and Bachtiar, 1995). The main problem is not that embryogenic tissue from primary explants can be obtained, but the large scale multiplication of this tissue by secondary embryogenesis. For this purpose, either tissue consisting of torpedo shaped embryos or mature embryos can be used. Multiplication of torpedo shaped embryos is highly genotype dependent, while multiplication of mature embryos is largely genotype independent (Raemakers, 1993). Both primary and secondary somatic embryogenesis are characterized by the formation of propagules with a bipolar structure. These bipolar torpedo shaped embryos are already formed on the auxin supplemented step 1 medium. Therefore, Taylor

et al., (1995) proposed the term organized embryogenesis. Organized cells are defined as a group of actively dividing cells, having the tissues and organs formed into a characteristic unified whole (Walker, 1989).

A less organized type of somatic embryogenesis was developed by Taylor *et al.* (1995). With continuous selection, organized embryogenic tissue cultured on a Gresshoff and Doy (1972) medium salts and vitamins supplemented with 10 mg/l Picloram (GD2) converted gradually into a less organized tissue. This tissue consisted of a callus-like mass of (pro-)globular embryos which was very friable. Therefore, this tissue was called friable embryogenic callus (FEC). The cells in FEC are continuously in a state where they break away from group control and because of that they are not organized into a unified structure. FEC is maintained on a medium consisting of Gresshoff and Doy (1972) vitamins and salts, 7 g/l Daichin agar, 20 g/l sucrose and 10 mg/l Picloram (solid GD2). Every three weeks the friable embryos were subcultured on the above mentioned medium. In order to initiate liquid suspension cultures 0.5 g of friable embryos was transferred in a flask of 200 ml with 50 ml of liquid medium supplemented with Schenk and Hildebrandt (1972) salts and vitamins, 60 g/l sucrose and 10 mg/l Picloram (liquid SH6). The medium was refreshed every 2-7 days and after 14 days the content of each flask was divided over 5 new flasks. The pH was adjusted to 5.7 before autoclaving. The temperature in the growth chamber was 30°C, the photoperiod 12 hours and the irradiance 40 $\mu molm^{-2}s^{-1}$. Suspension cultures were initiated by culturing FEC in Schenk and Hildebrandt (1972) medium supplemented with 6% (w/v) sucrose and 10 mg/l Picloram (SH6). Every 2-3 days this medium was refreshed.

To keep a culture in a highly friable state the FEC has to be sieved once in two months. In practice the part of the FEC which will go through a sieve with a mesh of 1 mm^2 will be used for subculture.

FEC will almost never form torpedo shaped embryos on the GD2 or in SH6 medium. Torpedo shaped and subsequent mature embryos are formed if

FEC is cultured on maturation medium. Maturation medium consist of Murashige and Skoog (1962) salts and vitamins, and 1 mg/l picloram. This maturation medium was refreshed every 3 weeks.

Mature embryos could be induced into secondary somatic embryogenesis by culturing on MS20 medium supplemented with 2,4-D, picloram, Dicamba or NAA. Primary and secondary somatic embryogenesis are relatively easy to establish in a wide range of genotypes (see Table 1), while FEC is for the time being restricted to a few genotypes. The prospect of FEC for a new system of somatic embryogenesis and genetic transformation is promising, although further research is needed to make this system applicable to more genotypes. Essential for this process is the availability of high quality organized tissue and the ability of this tissue to convert into FEC. Taylor *et al.* (1995) "used organized embryogenic tissues" which were multiplied at the torpedo shaped state to initiate FEC. In this case two steps (initiation of organized. tissue and conversion into unorganized tissue) are determinative for the successful initiation of FEC. Both steps are genotype dependent. If organized tissue is multiplied in the mature state as described by Raemakers (1993) then only the ability of this tissue to convert into FEC is a determinative step to initiate FEC. It remains to be investigated whether or not organized tissue can be used as starting material. If organized tissue cannot be used, then this tissue should be first multiplied in the immature state before it can be used to initiate FEC. This is readily accomplished by, either culturing explants at a high density or by reducing the cyclic duration.

FEC lines have been obtained in R60, R90, M7, TMS60444 and Adira 4 (Raemakers et al., 2000, 2001). Plant regenration from FEC has been achieved in R60, R90, M7, TMS60444 and Adira 4 (Raemakers et al., 2000, 2001). Taylor et all. (2000) has obtained FEC lines in the genotypes line 2, M.col1505, TMS90853, Kataoli and Bonoua Rouge.

Regeneration of plants from protoplasts

Isolation of protoplasts

For protoplast isolation both FEC cultured on solid GD2 or liquid SH6 can be used. However, the highest yield of protoplasts was obtained from FEC which has been cultured for 1 to 3 weeks in liquid SH6.

Two gram of FEC was placed in Petri dishes (∅ 9 cm) containing 10 ml of cell wall digestion solution. Cell wall digestion solution consisted of a mixture of cell wall degrading enzymes; 10 mg/l pectolyase, 10 g/l cellulose, 200 mg/l macero enzym growth regulators (NAA 1 mg/l, 2,4-D 1 mg/l, Zeatin 1 mg/l); major salts (368 mg/l $CaCl_2$; 34 mg/l KH_2PO_4; 740 mg/$KN0_3$; 492 mg/l $MgSO_4.7H_20$); minor salts (19.2 mg/l NA-EDTA; 14 mg/l $FeSO_4.7H_20$) and osmoticum (91 g/l D-mannitol) and 0.5 g/l MES. The cell wall degrading enzymes cellulase (1-10 g/l) plus Macerozyme (200 mg/l) were successful for protoplast isolation. The extra addition of Pectolyase (0.001-0.01 g/l) and/or Driselase (0.02 g/l) increased the yield of protoplasts. After 18 h of incubation, 10 ml of washing medium was added to the solution. Washing medium with an osmolarity 0.530 mOsm/kg consisted of major salts (see cell wall digestion solution), 45.5 g/l mannitol and 7.3 g/l NaCl. The digested tissue was filtered through a 73 μM pore size filter (PA 55/34 Nybolt - Switzerland) into a 250 ml beaker glass. The filtrate was divided equally over two 12 ml conical screw cap tubes, and centrifuged at 600 rpm for 3 min. (Mistral 2000). The washing procedure was repeated once after removal of the supernatant. The protoplast solution was resuspended by floating on 9.5 ml solution containing major and minor salts (see cell wall digestion solution) and 105 g/l sucrose. The pH was 5.8 and the osmolarity 0.650 mOsm. The solution with protoplasts was allowed to equilibrate for 5 minutes before 0.5 ml of washing medium was gently added on the top. After centrifugation at 700 rpm for 15 min. (Mistral 2000), the protoplasts ware concentrated in a band between the sucrose and washing

medium. The protoplast layer was harvested with a pasteur pipette and the yield was counted in a standard haemocytometer chamber.

Protoplast culture

Protoplasts ware cultured in media solidified with agarose 0.2% w/v (Dons en Bouwer, 1986) in petri dishes containing 10 ml of the same liquid medium. The following media resulted in the formation of micro callus:

- TM2G medium (Wolters *et al.*, 1991) supplemented with only auxins (0.1-10 mg/l NAA or 0.1-10 mg/l Picloram, or 0.1-10 mg/l IAA, or 0.1-10 mg/l 2,4-D, or 0.1-10 mg/l Dicamba, or 0.1-10 mg/l, or 0.1-10 mg/l) or auxins plus cytokinins (0.01-1 mg/l zeatin, 0.01-1 mg/l 2-iP, 0.01-1 mg/l BA, 0.01-1 mg/l TDZ, 0.01-1 mg/l kinetin).

- medium A (Murashige and Skoog (1962) salts and vitamins, 4.5 g/l myo-inositol, 4.55 g/l mannitol, 3.8 g/l xylitol, 4.55 g/l sorbitol, 0.098 g/l MES, 40 mg/l adeninsulphate and 150 mg/l caseinhydrolysate, 0.5 mg/l d-calcium-panthotenate, 0.1 mg/l choline-chloride, 0.5 mg/l ascorbic acid, 2.5 mg/l nicotinic acid, 1 mg/l pyridoxine-HCl, 10 mg/l thiamine-HCl, 0.5 mg/l folic acid, 0.05 mg/l biotine, 0.5 mg/l glycine, 0.1 mg/l L-cysteine and 0.25 mg/l riboflavine and 59.40 g/l glucose) supplemented with only auxins (0.1-10 mg/l NAA or 0.1-10 mg/l Picloram, or 0.1-10 mg/l IAA, or 0.1-10 mg/l 2,4-D, or 0.1-10 mg/l Dicamba plus cytokinins (0.01-1 mg/l zeatin, 0.01-1 mg/l 2-iP, 0.01-1 mg/l BA, 0.01-1 mg/l TDZ, 0.01-1 mg/l kinetin).

The media were refreshed every 10 days, by replacing 9 ml with fresh medium. After two months of culture in the first medium, high quality FEC was selected and either culture for further proliferation or for maturation. For proliferation FEC was transferred to Gresshoff and Doy (1974) medium supplemented with 40 g/l sucrose, 7 g/l Daichin agar and 2 mg/l picloram (GD4). After 3 weeks the FEC was transferred to a Gresshoff and Doy medium supplemented with 20 g/l sucrose, 7 g/l agar and 10 mg/l Picloram (GD2). Suspension cultures ware initiated by transferring 1.0 g of FEC to liquid

SH6% medium supplemented with 10 mg/l Picloram. Two weeks later the suspension was divided over new flasks with an initial packed cell volume of 1.0 ml.

After 2 months of culture, 10^4 protoplasts cultured in TM2G supplemented with 0.5 mg/l NAA and 1 mg/l Zeatin at a density of 10^5/ml produced 1058 micro-calli, whereas 10^4 protoplasts cultured at a density of 10^6/ml only produced 64 micro-calli.

Replacing TM2G medium with medium A reduced at both densities the number of micro-calli significantly. At this stage at least three types of calli could be distinguished. One type consisted of globular shaped embryos which were mostly observed in protoplasts cultured at a density of 10^6. Some of them developed cotyledon like structures, light green in color. However, these embryos could not be germinated properly. Another type was fast growing and consisted of a large compact callus, they were observed in protoplast cultures of both densities. This callus never developed embryos. The third type was highly friable callus and was observed at both densities. At a density of 2-5 x 10^5 (medium TM2G) about 60% of the calli were friable and embryogenic. The FEC was either subcultured for further proliferation or for maturation.

Proliferation of FEC derived from protoplasts

Following selection of FEC, 0.1 g of it cultured for three weeks on GD 4 plus 2 mg/l Picloram increased into 0.7 g of tissue. More than 95% of the tissue consisted of high quality FEC. Subsequently, this tissue was maintained by subcultures of three weeks on GD2 medium supplemented with 10 mg/l Picloram. To initiate suspension cultures FEC was transferred to liquid medium. The increase in packed cell volume (PCV) of this material was slightly higher than that of the original material (data not shown).

Maturation of FEC derived from protoplasts

In an attempt to induce maturation of embryos, FEC isolated after two months of culture in TM2G was cultured on maturation medium. Maturation medium consisted of Murashige and Skoog (1962) salts and vitamins, 10 g/l Daichin agar, 0.1 g/l myo-inositol, 20 g/l sucrose, 18.2 g/l mannitol, 0.48 g/l MES, 0.1 g/l caseinhydrolysate, 0.08 g/l adenine sulphate, 0.5 mg/l d-calcium-panthotenate, 0.1 mg/l choline chloride, 0.5 mg/l ascorbic acid, 2. Mg/l nicotinic acid, 1 mg/l pyridoxine-HCl, 10 mg/l thiamine HCl, 0.5 mg/l folic acid, 0.05 mg/l biotin, 0.5 mg/l glycine, 0.1 mg/l L-cysteine, 0.25 mg/l riboflavine and 1 mg/l picloram. This maturation medium was refreshed every 3 weeks.

On this medium there is a gradual shift from proliferation to maturation. As a result the packed cell volume had increased with a factor 4 after two weeks of culture in liquid maturation medium. Also after transfer to solid maturation medium there is proliferation. After two weeks on solid medium most of the embryos had reached a globular shape and only a few of these globular embryos developed further. The first torpedo shaped embryos became visible after one month of culture on solid maturation medium. The number of mature and torpedo shaped embryos was not correlated with the plating efficiency but with the density of the initially cultured protoplasts. No such embryos were obtained if protoplasts were cultured on TM2G without growth regulators. The highest number of mature and torpedo shaped embryos was formed from protoplasts cultured on TM2G supplemented with 0.5 mg/l NAA and 1 mg/l Zeatin. If NAA was replaced by Picloram then the number of torpedo shaped and mature embryos was significantly lower (Table 2). From the tested Picloram concentrations 2 mg/l gave the best results. After 3 months of culture between 60 and 200 torpedo shaped and mature embryos were isolated per agarose drop. Torpedo shaped embryos became mature at high frequency if they were cultured on fresh maturation medium or on MS2 plus 0.1 mg/l BAP.

Secondary somatic embryogenesis and germination of mature embryos derived from protoplasts

Only a few torpedo shaped embryos formed secondary embryos if cultured on liquid or solid MS2 medium supplemented with 10 mg/l NAA or 8 mg/l 2,4-D (data not shown). Mature embryos were better explants for secondary embryogenesis. In both liquid and solid medium 2,4-D was superior for induction of secondary embryogenesis as compared to NAA. If mature embryos were first cultured in 2,4-D and than in liquid NAA the response was comparable with culture in 2,4-D alone. Also embryos which first had undergone a cycle of secondary somatic embryogenesis in medium with 2,4-D, produced highly efficient secondary embryos in MS20 supplemented with 10 mg/l NAA.

The germination of cyclic or secondary somatic embryos, induced in liquid medium by the auxins 2,4-dichlorophenoxyacetic acid (2,4-D) or naphthalene acetic acid (NAA), was compared. In all genotypes desiccation stimulated normal germination of NAA induced embryos. However, the desiccated embryos, required a medium supplemented with cytokinins such as benzytaminopurine (BAP) for high frequency germination. The morphology of the resulting seedling was dependent on the concentration of BAP. With 1 mg/l BAP plants with thick and short taproots and branched shoots with short internodes were formed. With 0.1 mg/l BAP the taproots were thin and slender and the shoot had only one or two apical meristems. If the embryos were desiccated sub-optimally, higher concentrations of BAP were needed than if the embryos were optimally desiccated to stimulate germination. Also desiccated embryos which were cultured in the dark required a lower concentration of BAP and, furthermore, these embryos germinated faster than embryos cultured in the light. Complete plants were obtained four weeks after the start of somatic embryo induction. 2,4-D induced embryos showed a different response. In only one genotype desiccation enhanced germination of 2,4-D induced embryos and in three other genotypes it did not. In all genotypes

desiccation stimulated root formation. Embryos cultured in the dark formed predominantly adventitious roots, whereas embryos cultured in the light formed predominantly taproots.

Gene transfer systems

Over the past years several transfer techniques of DNA to plant protoplasts have been developed such as silicon fibers (Kaeppler *et al.*, 1990), microinjection (De Laat and Blaas, 1987) and electrophoresis (Griesbach and Hammond, 1993). The most commonly used and potentially-applicable ones are Agrobacterium-mediated gene delivery, microprojectile/particle bombardment and protoplast electroporation.

The Agrobacterium tumefaciens DNA delivery system is the most commonly used technique. It probably relates to the first invention of DNA delivery in plants by this method. Initially it was limited to Kalanchoe and Solanaceae, particularly tobacco. Nowadays, the use of Agrobacterium-mediated transformation has changed dramatically, it is possible to transform a wide range of plants including the most important monocots like maize and rice with a limitation in monocots (reviewed by Wordragen and Dons, 1992).

Although cassava is a host for Agrobacterium it has proven to be not highly amenable to it.

FEC from cassava has also been transformed efficiently via Agrobacterium tumefaciens (Raemakers et al., 2000, Schreuder et al., 2001). The method described by Raemakers et al., 2000 and Schreuder et al., 2001 has been used successfully to produce genetic modified plants from the genotypes Adira 4 and TMS604444 carrying the antisense gbss gene under control of the CaMV promoter.

In principle protoplasts are the most ideal explants for DNA delivery. They can be cultured as single cells that produce multicellular colonies from which plants develop. Plants derived from protoplasts are generally clonal in origin. This provides a useful tool for any transformation system, because it

will eliminate chimerism in transgenic plants. The use of protoplasts is, however, hampered by the regeneration system which is highly species dependent. For transformation, protoplasts can be used in conjunction with PEG to alter the plasma membrane which causes reversible permeabilization that enables the DNA to enter the cytoplasm as was demonstrated, for example, in *Lolium multiform* (Potrykus *et al.*, 1985) and *Triticum monococcum* (Lörz *et al.*, 1985). Another technique to increase the permeability of plasma membranes and even cell walls to DNA is by electroporation (for review see Jones *et al.*, 1987). In this method electrical pulses enable the DNA to enter the cells. Rice was the first crop in which fertile transgenic plants resulted from protoplast electroporation (Shimamoto *et al.*, 1989).

The use of particle bombardment or biolistics to deliver foreign DNA provides an alternative method in cassava transformation. Particle bombardment is the only procedure capable of delivering DNA into cells almost in any tissue. The first transgenic plant obtained by using this method was in tobacco (Klein *et al.*, 1989). Following this successful transformation method, particle bombardment is widely used in plants which are less amenable to *Agrobacterium* infection, particularly monocots. Improvement of several DNA delivery devices to accelerate the particle (microprojectile) has resulted in the most recent model the Biolistic ™ PDS-1000 (Blo-Rad Laboratories, Richmond, Ca). Those devices are available commercially, however the price is relatively high at present. Tungsten or gold particles, coated with DNA, are commonly used as microprojectiles to deliver DNA into the target tissue (reviewed by Songstad *et al.*, 1995).

Selection and reporter genes used in genetic modifications

To be able to identify transformed cells, the gene of interest is coupled to a selectable marker gene. This marker gene is necessary to select transformed cells. Selection can be based on a visual characteristic of the transformed cell/tissue. An example is the luciferase gene isolated from the firefly. Plant

cells expressing this gene and supplied with substrate (luciferin) will emit light which can be detected with special equipment (Ow *et al.*, 1986). Another way to select transformed tissue is the introduction of a gene which encodes resistance to antibiotics or herbicides (Thompson *et al.*, 1987; Gordon-*Kamm et al.*, 1990).

A number of antibiotics and herbicides has been used as selective agent in plant transformation. In cereals resistance to the herbicide phosphinothricin (PPT) was chosen for the selection of transgenic plants (Cao *et al.*, 1990). In *Carica papaya* (Fitch *et al.*, 1994), *Vitis vinifera* (Nakano *et al.*, 1994; Scorza *et al.*, 1995), maize (Rhodes *et al.*, 1988) and rice (Chen *et al.*, 1987) the neomycine phosphothansferase (NPTII) gene, which confers resistance to kanamycin and related antibiotics (Fraley *et al.*, 1986), was used as a selectable marker.

In cassava all above-mentioned systems of selection can be used, however PPT based selection has as advantage that it improves the ability of FEC to form mature embryos and in this way increase plant regeneration.

The following part describes how cassava plants genetically were modified with the aim to produce cassava plants with a high amylopectin content in their tuberous roots.

Isolation of the cassava gbss gene and construction of a plant transformation vector

In a previous study the gbss gene was isolated from cassava using the potato gbss gene (Visser et al., 1989) as probe (Salehuzzaman et al., 1993). The gbss gene was subcloned in anti-sense orientation between the potato gbss promoter (Visser et al., 1991) and the nopaline synthase terminator in pUC19 resulting in the vector pAG61 (Salehuzzaman et al., 1993). Also other preferably tuberous root specific promoters such as protein synthesis elongation factor 1-alpha from cassava (Suhandono et al., 2001), cassava gbss

or more general promoters such as CaMV can be used to direct expression of genes such as gbss.

The complete luciferase gene (BglII fragment) was isolated from pJIT100 (Guerineau et al., 1993) and inserted in the BamHI site of pAG61. This resulted in two different vectors: pGBSSas2 and pGBSSas7 (difference between the two vectors was the orientation of the luciferase and antisense gbss genes to each other). Both constructs were used successfully to produce cassava plants with a high amylopectin content.

Plant material and tissue culture media used

Plants of the genotype TMS60444 were maintained by monthly subculture of one node cuttings on medium supplemented with Murashige and Skoog (1962) salts and vitamins and 40 g/l sucrose (MS4). Friable embryogenic callus (FEC) lines were initiated as follows:

-isolation of meristems or immature leaves from donor plants

-cultured of meristems/leaves on MS40 supplemented with 6 mg/l NAA and 6 mg/l Picloram

-isolation of compact embryogenic tissue and cultured on a medium supplemented with Gresshoff and Doy (1974) salts and vitamins, 60 g/l sucrose and 10 mg/l Picloram (GD6).

-isolation of FEC (small clumps of aggregated, spherical units) which were cultured on GD6 medium. FEC was maintained by a 3 weeks subculture on GD6 medium. Liquid cultures were initiated by transferring 0.5 g of FEC into flask of 200 ml with 50 ml of liquid medium supplemented with Schenk and Hildebrandt (1972) salts and vitamins, 60 g/l sucrose and 10 mg/l Picloram (SH6). The medium was refreshed twice a week and after 2 weeks the content of a each flask was divided over 5 new flasks. The flasks were cultured on a rotary shaker (LAB-line Instruments Inc. Model 3519) at 120 rpm.

Coating of DNA on the particles.

A method adjusted from Cabe et al. (1988) was used to coat DNA on the particles. Eighty µg of DNA (isolated using WizardTM Maxipreps DNA purification system of Promega from the vectors pGBSSas2 and pGBSSas7) was mixed with 10 mg of gold particles (1.6 µm, BioRad), 30 µl 5 M NaCl, 5 µl 2 M tris HCl pH 8.0, 965 µL H2O, 100 µl 25% PEG 1550, 100 µL 0.1 M spermidine and 50 µl 2.5 M CaCl2. After centrifugation the pellet was resuspended in 10 ml of absolute alcohol and briefly sonificated. Hundred sixty µL of the suspension was pipetted in the hole of a macrocarrier holder placed up side down on a macrocarrier. After 5 minutes the macro carrier holder was taken away. The macrocarrier covered with a thin layer of gold beads was dried in an oven (10 minutes, 40 °C) and used for bombardment.

Bombardment of FEC and selection of transgenic plants

FEC cultured in liquid SH6 medium for at least 5 weeks, was sieved (mesh 1 mm) and collected. Hundred mg of FEC was spread on GD6 medium and bombarded using the BioRad PDS-1000He biolistic device (helium pressures 1100 p.s.i., 0.5 cm distance between rupture disc and the macrocarrier and between macrocarrier and the stopper plate, 5.0 cm distance between stopperplate and FEC, 27 inches Hg vacuum).

In total 212 and 184 Petri dishes with FEC from TMS60444 were bombarded with construct GBSSas2 or GBSSas7 respectively. After bombardment the bombarded FEC was cultured in plastic pots filled with liquid SH6 medium. Two weeks later the FEC was collected on solid GD6 medium and assayed for luciferase activity. Each luciferase (LUC) spot was subcultured as an individual line. In total 186 luc spots were produced with construct GBSSas2 and 222 with GBSS7. Because it was not possible to locate precisely which FEC unit contained LUC activity and to avoid loss of transgenic tissue, the tissue in a radius of 0.5-1 cm around the LUC spot was transferred in liquid SH6 medium. Two weeks later the FEC was assayed for

luciferase activity. Lines without luciferase activity were discarded and lines with 4 or more spots were used for subclump division The lines with 1-3 spots were transferred again to liquid Sh6 medium and two weeks later the lines in which the number of spots had increased to 4 or more were used for sub clump division; the others were discarded.

Forty four lines were obtained from the bombardments with pGBSSas2 and 40 lines from the bombardments with pGBSSas7.The transgenic tissue was isolated and purified via a process called subclump division (Raemakers et al., 2000). Subclump division started with subculturing the tissues around (0.5 to 1 cm diameter) a luciferase positive spot. The tissue was divided as fine as possible on GD6 medium. Two weeks later the Petri dish was covered with small clumps of FEC tissue. Only the LUC positive clumps were subcultured. For this the clumps were divided in subclumps and cultured on GD6 medium. This selection procedure was repeated 1-2 times more before the tissue was cultured for plant regeneration. For this the 84 FEC lines were subcultured every 2-3 weeks for 10-12 weeks on maturation medium (MS4 supplemented with 1 mg/l Picloram). Torpedo shaped somatic embryos were isolated from the FEC and cultured on MS4 supplemented with 0.1 mg/l BAP, which allowed further maturation. Mature somatic embryos were first cultured for two weeks in liquid and hereafter in solid germination medium (MS4+1 mg/l BAP). Plants were rooted on MS4 medium. Plants were obtained from 31 of the 44 GBSSas7 cultured lines and 27 of the 40 GBSSas7 cultured lines. These plants were first grown on Murashige and Skoog medium supplemented with 8% sucrose to allow starch disposition in the stems of the cassava plants (Salehuzzaman et al., 1994). The amylose/amylopectin ratio was visualised by iodine staining of cross sections of the in vitro thickened stems with Lugol's solution (I2:KI). The stained stem sections were visualised microscopically. In total 9 lines (3 from GBSSas2 and 6 from GBSSas7) yielded plants with an altered staining pattern, meaning that said plants had a high amylopectin content in the stems. These plants

were transferred to the greenhouse to allow formation of tuberous roots. Three months later the roots were peeled and the central cylinder of the storage root was grounded in a laboratory blender in water with a small amount of $Na_2S_2O_5$. The slurry was transferred for starch isolation to a Sanamat. The water-starch granules solution was transferred to centrifuge tubes and centrifuged. The starch was dried at 20 degrees Celsius for 3 days.

The amylopectin/amylose content was determined using the protocol described by Hovenkamp-Hermelink et al. (1988).

In total 2 lines (one from GBSSas2 and one from GBSSas7) had yielded plants which in both tests had a high amylopectin content. One year later the same plants were transferred again to the greenhouse. Starch of in total 30 plants of the two lines were analysed via iodine staining and via spectrophotometrie. All plants had starch with a high amylopectin content. At the same moment starch was isolated from more than 3000 plants. This starch was analysed in three different laboratories and again it was shown that the starch contained a high percentage amylopectin.

Materials & Methods

Samples

Tapioca starch	control sample
Amylopectin tapioca starch	AFC3KD from Wageningen
Potato starch	Oostermoer 1998
Amylopectin potato starch	Oostermoer 1996
Corn starch	Meritena A
Waxy corn starch	Meritena 300

Methods

Nitrogen content (N_{total})

ISO 5378 (1978), Starch and derived products - Determination of nitrogen by the Kjeldahl method - Spectrophotometric method.

Particle size distribution

The particle size is measured with a Coulter Multisizer II, calibrated with Coulter calibration standard P.D.V.B. Latex lot F.34, diameter measuring tube 140 µm, number of measured classes 256, measuring range 3,1-107,7 µm (potato starches) and 2,8-82,0 µm (other starches). The samples are suspended in isotonic salt solution (DiluidTM azid free, J.T. Baker) and homogenized in a ultrasonic bath (Branson 5510).

Differential scanning calorimetry

Differential scanning calorimetry experiments are performed using a Perkin-Elmer DSC-7. At least 10 mg of starch and 40 mg of demineralised water are put in a stainless steel DSC-pan to obtain 80% water content thereby taking the water content of the starch into account. The DSC-pan is hermetically sealed and stored one night at room temperature to equilibrate. Next day, the sample is heated from 5 to 130 °C with a rate of 10 °C/min.

Dry substance content

ISO 1666 (1997), Starch and derived products - Determination of moisture content - Oven drying method.

Ash content

ISO 5984 (1978), Animal feeding stuffs - Determination of crude ash content.

Intrinsic viscosity (IV)

The intrinsic viscosity is determined in a known manner with a Ubbelohde viscosity meter with 1 M sodium hydroxide as solvent and expressed in g/dl. As described in H.W. Leach in Cereal Chemistry, vol. 40, page 595 (1963).

Phosphor content (P)

ISO 3946 (1982), Starch and derived products - Determination of total phosphorous content - Spectrophotometric method.

Viscosity behavior

Determined with a Rapid Visco Analyser (RVA) from Newport Scientific. The measurements are carried out in a 6% concentration in demineralized water at 400 rpm. Temperature cycle: 2 min at 45°C, heating to 90° at 14° per min, 5 min at 90° and cooling to 30° at 14° per min.

Chain length distribution

The amylopectin starch is debranched with isoamylase, the resulting lineair alfa-1,4 malto-oligosaccharides are measured with High Performance Anion Exchange Chromatography with a Pulsed Amperometric Detection System.

Results

The results are shown in the following tables.

Table 1: Chain length distribution (weight average)

	Cassava	Amylo-pectin Cassava	Maize	Waxy Maize	Potato	Amylo-pectin Potato
DP 6 t/m 9	4.1 (5.9)	4.6 (5.7)	2.4 (3.9)	3.3 (3.7)	2.1 (3.7)	2.6 (3.9)
DP 6 t/m 12	14.7 (21.7)	16.4 (20.4)	11.2 (18.2)	15.3 (17.0)	7.6(13.5)	9.5 (14.1)
DP 13 t/m 24	31.9 (46.6)	36.9 (46.0)	33.2 (54.0)	46.2 (51.4)	29.2 (51.7)	33.3 (49.4)
DP 25 t/m 40	14 (20.4)	17.5 (21.8)	13 (21.1)	19.2 (21.4)	12.6 (22.3)	14.9 (22.1)
DP >40	7.8 (11.4)	9.4 (11.7)	4.3 (7.0)	8.9 (9.9)	7.4 (13.1)	9.8 (14.5)
Recovery(%)	68.5 (100)	80.2 (100)	61.5 (100)	89.9 (100)	56.5 (100)	67.4 (100)

Table 2: Various parameters

Parameter	Waxy Maize	Amylo-pectin cassava (ACS)	Amylo-pectin potato (APS)	Character ACS
RVA				
Tg	62.3 °C	61.7 °C	63.3 °C	Low Tg for ampec starch
Tg – Ttop	10.0 °C	5.9 °C	5.8 °C	Fast dissolving
Ttop visco	75 RVU	112 RVU	171 RVU	
Tend visco	48 RVU	87 RVU	77 RVU	
Brabender				
Tg	66 °C	60.5 °C	61.5 °C	Low Tg for ampec starch
Tg - Ttop	6.5 °C	6 °C	7 °C	Fast dissolving
Ttop visco	685 BU	990 BU	1560 BU	
T20'90	250 BU	290 BU	440 BU	
Tend visco	400 BU	495 BU	670 BU	
Amylose content		< 1%	< 1%	Schoch, detection level
Visco stability	Stable	Stable	Stable	No differences
Protein	0.41 mg/g	0.16 mg/g	0.10 mg/g	Clear solution
Phosphate	< 0.05 mg/g	0.06 mg/g	0.81 mg/g	Clear solution, viscosity level
Chain length distribution	21	27	28	

Table 3: Various parameters

	dry substance mg/g as is	ash mg/g ds	N_{total} mg/g ds	P mg/g ds	IV dl/g ds
tapioca	860	3.5	0.11	0.06	2.4
amylopectin tapioca	863	2.3	0.16	0.06	1.7
potato	844	4.7	0.13	0.85	2.4
amylopectin potato	856	4.7	0.10	0.81	1.8
maize	879	1.1	0.53	0.18	1.7
waxy maize	881	<1.0	0.41	<0.05	1.6

Table 4: Particle size distribution

	weight average	d_{10}	d_{50}	d_{90}
	µm	µm	µm	µm
tapioca	13.0	7.8	12.6	16.9
amylopectin tapioca	14.0	7.7	13.0	19.2
potato	42.9	23.0	42.4	63.3
amylopectin potato	45.0	23.2	44.7	67.0
maize	14.0	9.8	14.1	18.4
waxy maize	14.3	9.3	14.0	18.3

Table 5: Results of gelatinisation behaviour measured by DSC.

	T_{onset} (°C)	T_{peak} (°C)	T_{end} (°C)	ΔT (°C)	ΔH (J/g)
tapioca	60.2 ± 0.4	65.0 ± 0.6	72.8 ± 0.7	12.6 ± 0.3	19.19 ± 1.2
amylopectin tapioca	61.7 ± 0.3	67.6 ± 1.0	75.9 ± 0.6	14.2 ± 0.4	19.11 ± 2.2
potato	62.6 ± 0.3	67.3 ± 0.6	75.1 ± 0.9	12.5 ± 0.6	22.12 ± 2.7
amylopectin potato	63.3 ± 0.3	69.1 ± 0.4	75.2 ± 0.6	11.9 ± 0.5	24.04 ± 1.4
maize	67.4 ± 0.2	73.2 ± 0.3	79.3 ± 0.9	12.1 ± 0.9	15.92 ± 0.6
waxy maize	62.3 ± 0.3	72.3 ± 0.3	80.4 ± 0.2	18.1 ± 0.4	18.76 ± 0.8

Different properties of amylopectin cassava starch compared to waxy maize starch:

- Low Tg
- Fast dissolving starch (difference between Tgelatinization and Ttop)
- About 20 % higher viscosity
- Less protein, low impurity level

Properties amylopectin cassava starch compared to amylopectin potato starch

- Somewhat lower Tg (< 1 C)
- Fast dissolving starch, however the viscosity of APS is higher (delta C = 6 C – BU = 1400)
- About 30 % lower viscosity level
- Small granules
- Crystallinity

Table 6: Genotypes of cassava used for somatic embryogenesis.

	Indonesia	Nigeria	TMS90853	M. Co122,	Sao Paolo
	Adira 1	TMS50395	TMS30555	Zimbabwe	Thailand
	Tjurug	TMS60444	TMS30211	Line 11	R5
	Adira 4	TMS90059	TMS30395	M7	KU50
	Mangi 4	TMS30572	TMS30001	Venuzuela	R60
	Gading	TMS4(2)1244	Columbia	M.Ven77	R90
	Faroka	TMS60506	M. Col 1505	Brasil	R1

Table 7: Influence of light intensity during growth of donor plants in vitro on the number of leaf explants responding with the formation of mature embryos and the number of mature embryos per cultured leaf explant (#ME/CLE).

light intensity ($\mu Em^{-2}s^{-1}$)	number of explants	responding explants[a]	production (# ME/CLE[b])
40	48	18 b	1.7 b
28	48	26 ab	4.9 ab
8	48	31 a	6.6 a

[a,b] means with the same letter are not significantly different by respectively Chi-square test ($p<0.1$) and by LSD test ($p<0.1$)

Table 8: Influence of 2,4-D pretreatment on production of primary mature embryos (# mature embryos per cultured leaf explant isolated from *in vitro* plants), followed by the multiplication of mature embryos by secondary somatic embryogenesis in 11 Nigerian cassava genotypes and in M.Col22.

embryogenesis	primary[a]		secondary[b]
2,4-D pretreatment	no	yes	
M.Col22	3.5	9.4	13.5
TMS 30555	0	0.7	6.2
TMS 50395	0	< 0.1	5.3
TMS 60506	0	< 0.1	0
TMS 90059	0	< 0.1	7.2
TM S 30211	0	0	-
TMS 60444	0	1.1	9.9
TMS 30395	0	0.1	6.7
TMS 90853	< 0.1	0.2	8.2
TMS 4(2)1244	< 0.1	0	5.4
TMS 30001	0	0	-
TMS 30572	0	0	-

[a] average of three experiments (total 48-74 leaf expiants), [b] average of two experiments (total 24-48 ME explants).

Legend to figure 1

Figure 1: Schematic representation of somatic embryogenesis in cassava, including primary, secondary somatic embryogenesis, selection of friable embryogenic callus, maturation and desiccation followed by germination.

gd2= medium supplemented with Gresshoff and Doy salts (1974) and vitamins plus 20 g/l sucrose.

gd4= medium supplemented with Gresshoff and Doy salts (1974) and vitamins plus 40 g/l sucrose.

ms2= medium supplemented with Murashige and Skoog salts and vitamins plus 20 g/l sucrose.

pic= 10 mgl/l Picloram, NAA=10 mg/l naphthalene acetic acid, 2,4-D=8 mg/l, 2,4-dichiorophenoxy acetic acid.

sh6= medium supplemented with Schenk and Hildebrandt (1972) salts and vitamins plus 60 g/l sucrose.

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